Antigenic mapping of human low density lipoprotein with monoclonal antibodies

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Abstract Monoclonal anti-LDL antibodies were produced in a mouse spleen-myeloma system and purified by affinity chromatography on insolubilized low density lipoprotein (LDL). Five antibodies with different specificities could be distinguished by their immunoreactivities with chemically modified LDL preparations, and by their competition for binding to LDL. One of the antibodies inhibited the binding of ^{125}I-labeled LDL to the apoB,E receptors of cultured human fibroblasts. The same degree of inhibition was achieved using isolated Fab fragments. This antibody may bind to an antigenic site located near the cellular binding site of LDL-apoB.—**Tikkanen, M. J., R. Dargar, B. Pfleger, B. Gonen, J. M. Davie, and G. Schonfeld.** Antigenic mapping of human low density lipoprotein with monoclonal antibodies. *J. Lipid Res.* 1982. **23:** 1032–1038.

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Apolipoprotein B (apoB) is synthesized in the intestine, the liver, and perhaps the kidney (1). The hepatic forms are designated as B-100, B-74, and B-26 based on their relative apparent molecular weights. Intestinal apoB is designated as B-48 (2). ApoB is the recognition marker on lipoproteins for the cellular apoB,E receptors, although the roles of the various apoB species are unknown. The attempts to clarify the primary and three-dimensional structures of the apoB species using chemical methods have not been successful due to the unique insolubility of delipidized apoB in aqueous solutions and its aggregability. Using an immunologic approach, polyclonal antisera directed against LDL or apoB have been used to probe the surface structure of apoB in VLDL and LDL (3, 4). Differences in apoB immunoreactivity appeared as VLDL underwent lipolysis, suggesting that the antigenic determinants of apoB were altered during lipolysis. Lipolyzed VLDL also interacted more avidly with the apoB,E receptors on human fibroblasts, suggesting that some of the antigenic and receptor recognition sites of apoB may overlap.

In order to probe the structure and function of apoB further, we produced and isolated monoclonal antibodies directed against human LDL. Antibodies were found to be directed against five antigenic determinants of apoB. One of the antibodies inhibited the binding of ^{125}I-labeled LDL to the apoB,E receptor of cultured human fibroblasts.

METHODS

Production of monoclonal antibodies

Female Balb/cJ mice (Jackson Memorial Laboratories, Bar Harbor, ME) were immunized with human LDL (d 1.025–1.050 g/ml) isolated by ultracentrifugation from the plasma of a donor with homozygous familial hypercholesterolemia (5). LDL contained no albumin by immunodiffusion, and B-100, B-74 and B-26 were the only discernible proteins on SDS electrophoresis in 3% polyacrylamide (2). Fifty to 100 μg of holo LDL (EDTA-saline) emulsified in complete Freund's adjuvant was given first subcutaneously, followed by three to four boosts in incomplete adjuvant at intervals of 2–4 weeks. Splenic cells, $\sim 10^8$, were fused with $\sim 10^7$ SP2/0-Ag14 mouse myeloma cells using polyethylene glycol (6). Products from the two fusions were plated in 24-well cluster plates and grown overnight in Dulbecco's modified Eagle's medium containing 5% newborn calf serum and 10% agammaglobulinemic horse serum (DME-S). Then, fusion products were grown in DME-S containing 14 mg/l hypoxanthine, 0.176 mg/l aminopterin, and 3 mg/l thymidine (DME-S-HAT) for 3 weeks. Media were tested for antibody activity and cells from one well that contained antibody were cloned by dilution in 0.2% agar (in DME-S). After 2 weeks, selected clones were picked and expanded in T-flasks in DME-S, and those continuing to show antibody activity were expanded further as ascites tumors in Pristane (2-6-10-14 tetramethylpentadecane, Aldrich Chemical Co., Milwaukee, WI)-treated Balb/cJ mice.

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Binding assays using microtiter plates

Testing of hybridoma fluids was done on 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA). First, 150 μl of rabbit antihuman LDL antiserum was added to each well. After 16 hr at 23°C, wells were rinsed with PBS (0.16 M NaCl and 0.05 M phosphate, pH 7.4), filled with PBS-3% bovine serum albumin (PBS-BSA) for 4 hr, and rinsed again with PBS. Then each well received 130 μl of hybridoma culture media (sample), media from irrelevant fusion products ($blank_1$), or unspent DME-S ($blank_2$), plus 100,000 cpm of ^{125}I-labeled LDL and incubation was carried out overnight. Microtiter wells were rinsed with PBS, sliced, and counted in a Packard gamma spectrometer.

Binding of affinity-purified anti-LDL monoclonal antibodies to microtiter wells was assessed by coating wells with ^{125}I-labeled antibody solutions of differing concentrations overnight at 22°C. Wells then were rinsed repeatedly with PBS, sliced, and counted in a Packard gamma spectrometer. To assess binding of LDL to antibodies, microtiter wells were coated with 150 μl (750 ng) of affinity-purified antibody solutions overnight. After rinsing and blocking with PBS-BSA, increasing amounts (in 150 μl) of ^{125}I-labeled LDL (~50 cpm/ng) were added for 16 hr at 23°C. The wells then were rinsed again with PBS, sliced, and counted in a gamma counter. In competitive displacement assays using modified LDL preparations, the microtiter wells coated with affinity-purified monoclonal anti-LDL antibodies received increasing amounts of modified LDL and a constant amount of ^{125}I-labeled unmodified LDL. Plates then were processed as above. Competitive displacement of ^{125}I-labeled antibodies by nonlabeled antibodies was studied on microtiter plates coated with 150 μl of LDL (20 μg/ml) overnight at 23°C. After rinsing with PBS and blocking with PBS-BSA, wells received increasing amounts of nonlabeled antibodies and a constant amount (~100,000 cpm) of one ^{125}I-labeled antibody. Each antibody was labeled and, in turn, tested against the other "cold" antibodies. Plates were then processed as above.

^{125}I-labeling of antibodies was carried out using chloramine-T (7) and that of LDL was done using lactoperoxidase (8).

Purification of antibodies

Seven clones producing anti-LDL antibodies were processed. The antibodies were identified as IgG by immunoassay using class-specific antisera. Following precipitation of IgG from ascites fluid in 50% saturated $(NH_4)_2SO_4$, antibodies were isolated on an immunosorbent column (9) prepared from the immunizing LDL and CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The coupling of LDL to Sepharose was carried out in 0.1 M $NaHCO_3$ buffer, pH 8.3, (2 hr, 23°C) according to the directions of the manufacturer, using 25 ml of swollen gel and 64 mg of LDL. Columns (8 × 40 mm) (Chromaflex Disposable Columns, Kontes Glass Co., Vineland, NJ) containing 3.5 ml of gel were prepared, one for each antibody. Unbound protein was eluted with 0.9% NaCl (15 ml) and the specifically bound fraction (monoclonal antibody) was eluted with 0.1 M glycine-HCl, pH 2.5. The bound fraction was neutralized upon elution by adding 1.1 M Tris buffer, pH 8.6. Between 30–40% of sample protein was recovered in the bound fraction. Purified antibodies migrated as single sharp bands on cellulose acetate electrophoresis (Microzone Electrophoresis System, Beckman Instruments, Inc., Fullerton, CA) and formed three bands on isoelectric focusing (10).

Cell studies

Cultures of normal human fibroblasts and the quantitation of cell associated and degraded ^{125}I-labeled LDL were performed as described (11, 12). Cells were grown in Eagle's minimal essential medium (15% fetal calf serum) for 5 days and in 10% lipoprotein-deficient serum for 48 hr. At the start of the experiments, increasing amounts of antibodies purified by affinity chromatography, or their isolated Fab fragments, were added to the culture media, followed 5 min later by ^{125}I-labeled LDL (5 μg/ml). Incubations in triplicate were carried out at 37°C for 4 hr. At the end of incubations cells were washed, dissolved in 0.1 M sodium hydroxide, and aliquots were taken for determination of cell protein (13) and of counts. Results are expressed as ng of ^{125}I-labeled LDL bound/mg cell protein.

Preparation of Fab fragments

Fab fragments were prepared as follows (14): 1–2 mg of affinity-purified antibody was incubated at 37°C for 4 hr in 6 ml of 0.1 M phosphate buffer, pH 8.0, 2 mM Na_2EDTA, 10 mM l-cysteine, and papain, 10 μg/mg antibody (Sigma Chemical Company, Inc., St. Louis, MO). Separation of Fab from Fc fragments and partially digested antibody was achieved by chromatography on DEAE cellulose (DE 52; Whatman Ltd., Kent, England) (linear gradient, starting buffer 0.005 M phosphate, pH 8.0; limiting buffer 0.3 M phosphate, pH 8.0). Fab fragments immobilized on microtiter wells bound ^{125}I-labeled LDL, they migrated cathodally as a single arc on immunoelectrophoresis, and showed bands of ~26,500 and ~52,000 apparent molecular weight on SDS gel electrophoresis in the presence of 1% 2-mercaptoethanol (100°C for 2 min) (15).

TABLE 1. Binding of monoclonal antibodies to microtiter plates

Antibody Added	Antibody Bound					
	1	2	3	4	5	6
ng/well			*ng/well*			
250	23	17	18	29	29	66
500	30	20	22	35	32	75
750	38	23	25	41	39	84
1250	48	25	27	49	48	98
5000		32	33	69	62	142
10000		36	36	76	67	143

Microtiter plates were coated with the indicated amount of ^{125}I-labeled antibodies. Specific radioactivities 100 cpm/ng were obtained by mixing trace amounts of ^{125}I-labeled antibody with appropriate amounts of "cold" antibody. The amount of antibodies bound per well was calculated from cpm/well and known specific radioactivities. Results are means of duplicate wells, which agreed to within 3% of the means. Experiments carried out on two separate occasions yielded virtually identical results.

Antibodies: 1 = 457C4D4; 2 = 464B1B3; 3 = 464B1B6; 4 = 465B6C3; 5 = 465C3D1; 6 = 465D3D5.

Chemical modifications of LDL

Modifications of the lysyl and arginyl groups on LDL were carried out as follows. Acetoacetylation was performed using diketene (16, 17), reductive methylation with sodium borohydride and formaldehyde (16, 18), carbamylation with cyanate (16), and acetylation was performed according to Fraenkel-Conrat (19). Arginine residues were modified by 1,2-cyclohexanedione according to the modification of Mahley et al. (20) of the method of Patthy and Smith (21). Malondialdehyde-altered LDL was produced according to the method of Fogelman et al. (22). Products were analyzed by electrophoresis on agarose. Each lipoprotein yielded a single sharp band indicating that the modified LDL was homogeneous (4). While the extent of modification of amino acid residues was not quantified here, modifications carried out by others under similar conditions (16, 20) yielded alterations of 25–35% of the appropriate amino acid residues. One preparation of LDL isolated from the plasma of a patient with homozygous familial hypercholesterolemia was used for all of these studies. The individual chemical modifications were carried out in sufficiently large batches so that all of the antibodies were tested with a single batch of a given modified LDL. Modified and unmodified "standard" LDL were run in the same radioimmunoassays in five or six doses in triplicate. Results are given as percent of the unmodified LDL preparation, i.e., displacement curves of those preparations that are >100% were to the left of the standard, those <100% were to the right of the standard. Apparent contents were calculated from logit B/Bo vs. dose curves using a Hewlett-Packard programmable calculator and a program provided by the vendor (23, 24). Coefficients of variation of the triplicate points averaged ±7%.

RESULTS

Binding of monoclonal antibodies to microtiter plates and binding of LDL to antibodies

Similar amounts of affinity-purified antibodies were bound to plastic, except for antibody 465D3D5, which was bound to a much greater extent than the others (**Table 1**). When 750 μg of antibody/well was added, binding ranged from 23–84 μg/well. Antibody 457C4D6 was not tested. When microtiter wells were coated with 5 μg/ml solutions of the different affinity-purified antibodies, and increasing amounts of ^{125}I-labeled LDL were added, the binding of LDL increased and then leveled off at ~2.5 μg of LDL added per well. The half maximum binding of antibodies varied between 12 and 80 ng of LDL bound per well (**Fig. 1**). To obtain some quantitation of the LDL binding characteristics of these antibodies relative to each other, we calculated ratios of one-half maximum mass ^{125}I-labeled LDL bound/mass antibody bound per well (**Table 2**). Ratios ranged from 3.48 to 0.14, about 24-fold. How-

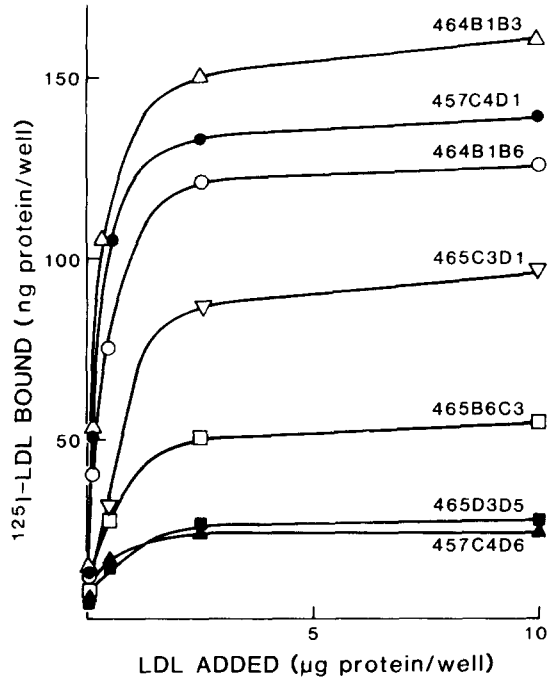

Fig. 1. Binding of LDL to antibodies. Microtiter wells were coated with 150 μl (5 μg/ml) of purified antibody solution overnight. After rinsing and blocking with PBS-BSA, increasing amounts (in 150 μl) of ^{125}I-labeled LDL (~50 cpm/ng) were added (16 hr at 23°C), rinsed again with PBS, sliced, and counted in a gamma counter. Triplicate determinations yielded coefficients of variation of ~5%.

TABLE 2. Binding of ^{125}I-labeled LDL by anti-LDL monoclonal antibodies

Binding	Antibodies[a] 1	2	3	4	5	6
	ng/well					
½ Maximum ^{125}I-LDL bound	68[b]	80	62	26	47	12
Antibodies bound	38[c]	23	25	41	39	84
^{125}I-LDL/bound antibody	1.79	3.48	2.48	0.64	1.20	0.14

[a] See Table 1 for designations of antibodies.
[b] See Fig. 1 for binding of ^{125}I-labeled LDL.
[c] See Table 1 (750 ng antibody/well added).

ever, the range of ratios was only 5-fold for five of the six antibodies tested. While these results are not rigorous determinations of affinities in bulk solutions, they do provide some ranking of the apparent LDL binding affinities of the antibodies on plastic microtiter plates.

Immunologic specificities of antibodies

In order to distinguish whether the antibodies had differing specificities vis-a-vis LDL, the binding of various modified LDL by the different anti-LDL antibodies was tested in competitive displacement assays (RIA) (**Table 3**). Increasing amounts of native or variously modified LDL preparations and constant amounts of ^{125}I-labeled LDL were added to microtiter plates that had been precoated with the different monoclonal antibodies. The apparent apoB contents of various LDL preparations differed from each other in all assays, e.g., when the LDL was analyzed with antibody 464B1B3, cyclohexanedione (CHD)-treated and reductively methylated (R-CH_3) LDL had 59 and 10% of the immunoreactivity of native LDL, respectively. The apparent apoB contents of the different LDL's were similar in assays containing antibodies 464B1B3 and 464B1B6. Similarly, the patterns of reactivity of LDL when assayed with antibodies 457C4D1 and 457C4D6 were nearly identical. However, the patterns of immunoreactivity differed with antibodies 457 and 464, and each of the three 465 antibodies also yielded unique patterns. Thus, five different patterns of reactivity were distinguished, one corresponding to antibodies 457C4D1 and 457C4D6, another to antibodies 464B1B3 and 464B1B6, and three additional ones corresponding to each of the three 465 antibodies.

The above data suggested that each of the five uniquely reacting antibodies bound at a different antigenic site on apoB. This was tested by assessing the ability of each antibody to compete with the other antibodies for binding to LDL. LDL was immobilized on microtiter plates, increasing amounts of unlabeled antibody and a constant amount of labeled antibody were added to the wells (**Fig. 2**). The binding of ^{125}I-labeled antibody 457C4D1 was markedly inhibited by increasing amounts of nonlabeled antibodies 457C4D1 and 457C4D6, but other antibodies did not inhibit at all or inhibited less effectively (Fig. 2a). The binding of ^{125}I-labeled 464B1B6 was inhibited by increasing amounts

TABLE 3. Immunoreactivity of monoclonal anti-LDL antibodies with chemically modified LDL

LDL Preparations	Antibodies: Apparent apoB Contents[a] (% of LDL protein) 457 C4D1	457 C4D6	464 B1B3	464 B1B6	465 B6C3	465 C3D1	465 D3D5
Native LDL	100	100	100	100	100	100	100
Acetyl-LDL	269	302	14	13	3	660	83
MDA-LDL		65		14	22	143	225
Aceto acetyl-LDL	90	95	26	27	16	100	46
R-CH_3-LDL	31	30	10	8	5	222	63
CHD-LDL	126	136	59	62	100	217	31
CB-LDL	205			38		188	339

[a] Apparent apoB contents were assessed by RIA. Results (% of LDL protein) were obtained by using unmodified LDL as standard. Coefficients of variation of these measurements, five or six doses in triplicate, averaged 7%.

Abbreviations: R-CH_3 = reductive methylation; CHD = cyclohexanedione; MDA = malondialdehyde; CB = carbamylated.

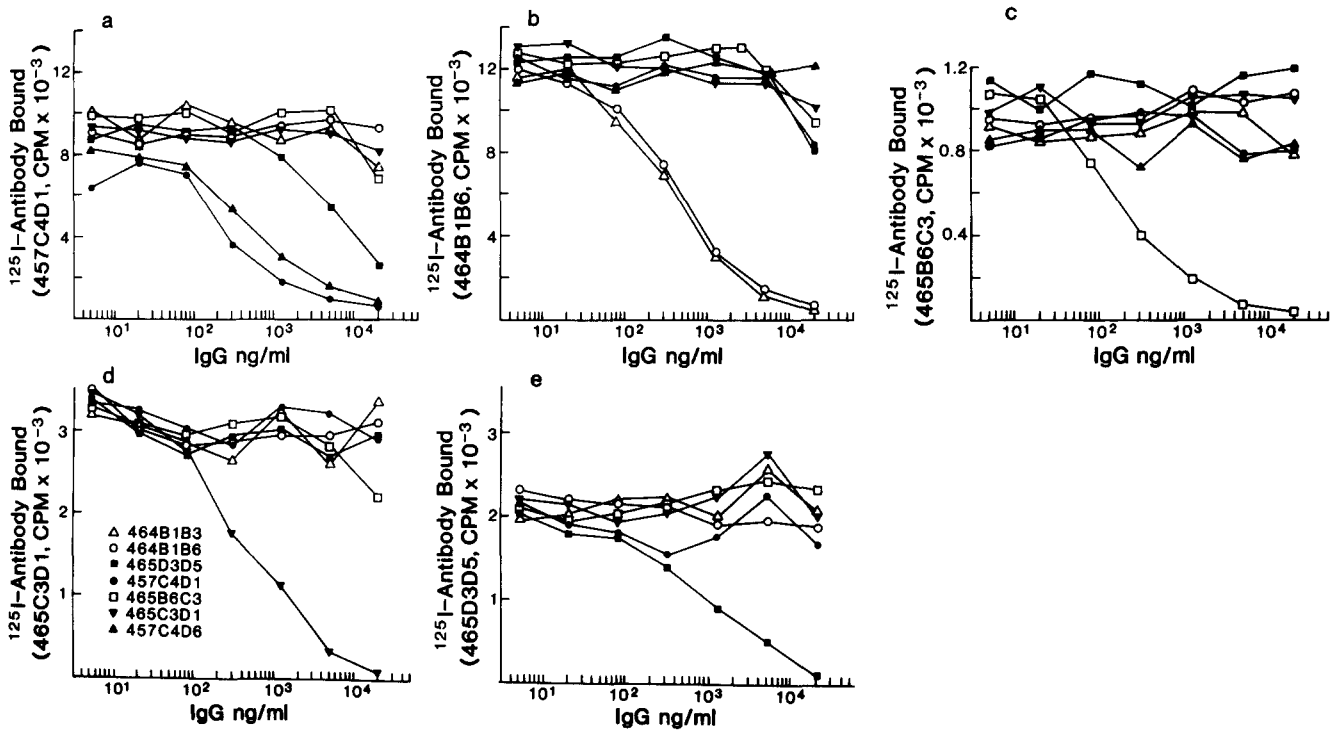

Fig. 2. Inhibition of binding of ^{125}I-labeled antibodies to LDL by unlabeled antibodies. Microtiter wells were coated with 150 μl of LDL (20 μg/ml) overnight at 23°C, rinsed with PBS, and blocked with PBS-BSA. Wells then received increasing amounts of unlabeled antibodies as indicated on the abscissas and a constant amount of ^{125}I-labeled antibodies (indicated on the ordinates). After incubation (16 hr at 23°) wells were rinsed again with PBS, sliced, and counted in a gamma counter. Determinations carried out in triplicate yielded coefficients of variation of ~5%.

of nonlabeled antibodies 464B1B3 and 464B1B6 but not by other antibodies (Fig. 2b). Similarly, the binding of ^{125}I-labeled antibodies 465B6C3, 465C3D1, and 465D3D5 was inhibited only by their respective nonlabeled parent compounds (Fig. 2c–e).

Inhibition by antibodies of binding of ^{125}I-labeled LDL to fibroblasts

The effect of various antibodies on the cellular binding and degradation of ^{125}I-labeled LDL was investigated by adding increasing amounts of purified antibody followed by a constant amount of the labeled lipoprotein to the culture media of cultured normal human fibroblasts (see Methods and legend to Fig. 3). Antibodies 464B1B3 and 464B1B6 (which exhibited similar binding to modified LDL and similar specificity in competition assays) both inhibited the cellular binding and degradation of ^{125}I-labeled LDL, whereas the other antibodies inhibited only at 100-fold greater doses, or not at all (**Fig. 3**). The incubations with antibodies were repeated on three other occasions with similar results. In a separate experiment, equivalent amounts of antigen-binding Fab fragments of antibody 464B1B6 were added to the system and they provided the same degree of inhibition (Fig. 3). Similarly, only antibodies 464B1B3 and 464B1B6 inhibited the binding of LDL to the apoB,E receptor in cultured human monocyte-macrophages (22, 25). None of the antibodies tested inhibited the binding (or degradation) of AcLDL by the so-called 'scavenger' receptor in the same preparations (data not shown). These studies suggest that the site on LDL, which recognizes apoB,E receptor, is similar for receptors present on fibroblasts or macrophages.

DISCUSSION

Seven monoclonal antihuman LDL antibodies were purified by affinity chromatography on LDL immobilized by coupling to CNBr-activated Sepharose 4B. The apparent LDL binding affinities of these antibodies varied over a 25-fold range (Tables 1 and 2).

A comparison of the specificities of these antibodies was performed in two types of experiments. First, chemically modified LDL was made to compete with ^{125}I-labeled unmodified LDL for binding to the antibodies. Five different patterns of immunoreactivity were distinguished (Table 3). With some antibodies all chemical modifications decreased the immunoreactivity of LDL, suggesting that at least some of the lysyl or arginyl residues may be part of or near the antigenic site defined

by the antibody. Alternatively, modification of the residues may have altered the conformation of the proteins so as to "mask" antigenic sites, whether or not the modification occurred near the antigenic site. Modification of LDL enhanced its reaction with some antibodies. This may be due to alterations in conformations that "unmasked" antigenic sites. In a second set of experiments, only labeled and homologous nonlabeled antibodies competed with each other for binding sites on immobilized LDL, but nonhomologous antibodies did not compete. These studies confirmed that antibodies with at least five differing specificities had been produced. Studies to localize the antigenic sites defined by these antisera to the B-100, B-74, B-48, and B-26 subspecies of apoB are in progress.

Antibodies 464B1B3 and B1B6 both inhibited the uptake and degradation of ^{125}I-labeled LDL by cultured normal human fibroblasts (Fig. 3) and monocyte-macrophages (not shown). As little as 0.25 μg of the antibodies was sufficient to inhibit ^{125}I-labeled LDL processing by 25%, 1 μg inhibited by ~50%, 5 μg by ~70%, and 50 μg by 70–80%. It is not clear why 100% inhibition was not achieved. Perhaps 5 min of preincubation was not long enough to achieve complete "occupancy" of ^{125}I-labeled LDL by antibodies. Had antibodies and ^{125}I-labeled LDL been preincubated for a longer time, it is possible more complete inhibition might have been obtained. Heterogeneity of ^{125}I-labeled LDL binding to antibody also could have affected the results. If some subpopulations of ^{125}I-labeled LDL were bound to the antibodies with lower affinity than the rest of the ^{125}I-labeled LDL preparations, the subpopulations could have been taken up by cells. Finally, it is possible that the residual processing of ^{125}I-labeled LDL (not inhibited by antibody) was due to "nonspecific" non-receptor-mediated binding and degradation of ^{125}I-labeled LDL by cells. We have no direct data on these points. Nevertheless, it is clear that the 464 antibodies exert considerable inhibition on the processing of ^{125}I-labeled LDL by cells. Other antibodies were considerably less active in this regard. For example, 50 μg of antibody 457C4D1 were required to produce the same degree of inhibition as was achieved by ~0.25 μg of the 464 antibodies, and antibody 457 was the most active inhibitor compared with antibodies 464. Since the apparent affinity of 464B1B3 probably is only twice that of 457C4D1, it is unlikely that differences in affinity account for the large difference in inhibiting capacity. Similarly, the one-third smaller apparent affinity of antibody 465C3D1 relative to antibody 464B1B3 is unlikely to account for the former's <1/200th inhibitory activity. It is more likely that the inhibition by 464 was related to the blocking of an antigenic site that is located close to the cellular apoB,E receptor recognition site.

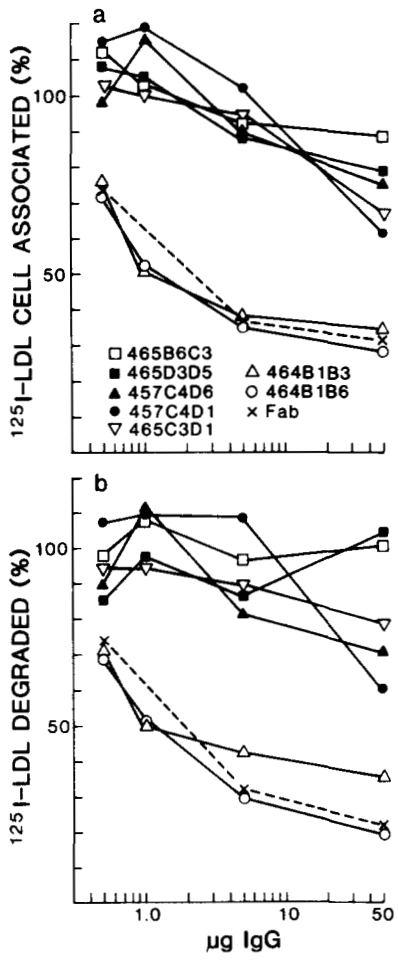

Fig. 3. The binding (a) and degradation (b) of ^{125}I-labeled LDL by cultured normal human fibroblasts. Cells were grown in Eagle's minimal essential medium (15% fetal calf serum) for 5 days and in 10% lipoprotein-deficient serum for 48 hr. At the start of experiments increasing amounts of affinity-purified antibody were added to the culture media. Five min later, 5 μg/ml of ^{125}I-labeled LDL (~100 cpm/ng) was added. At the end of incubations (4 hr at 37°C), cell-associated and degraded LDL counts were determined. In a separate experiment, equivalent amounts of antigen binding Fab fragments (⅔ of IgG mass) were used instead of whole antibodies. The results are expressed as percent of maximum binding obtained in the absence of antibodies. Determinations carried out in triplicate yielded coefficients of variation of ~10%.

The nearly identical inhibition obtained by the Fab fragments of antibody 464B1B6 strengthens this conclusion, because the small size of the Fab fragments lessened the likelihood that antibody inhibition was due to steric inhibition of binding of LDL to cells. Of course, direct proof that the antigenic determinant defined by antibody 464B1B3 (464B1B6) and the cellular recognition site overlap awaits the isolation of a fragment of apoB that specifically binds to the apoB,E receptor. Monoclonal anti-LDL antibodies may be useful for the isolation of apoB fragments that contain functionally important structures, such as the cellular apoB,E rec-

ognition site. Therefore, these antibodies may help to elucidate apoB structure.

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